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Research article

The effects of carbon and nitrogen sources on the growth of *Isaria tenuipes* (Peck.) Samson (DL0099) from Lam Dong, Vietnam

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Abstract

Isaria tenuipes (Peck.) Samson is a mushroom with high medicinal value. This study evaluated the effects of carbon and nitrogen sources on the mycelium growth and fruiting body formation of *I. tenuipes* (Peck.) Samson (DL0099) from Lam Dong, Vietnam in *in-vitro*. The results have determined that glucose with a concentration of 20~40 g/L was suitable for the growth of DL0099. Organic nitrogen sources (peptone, malt extract, and yeast extract) were suitable for the growth of *I. tenuipes* (DL0099), in which peptone 10 g/L was the most suitable for the mycelium density and colony diameter. The formation of fungal fruiting bodies was best in the medium supplemented with malt extract 10 g/L. The results will be beneficial to mushroom growers in biomass production and fruit body formation of the Vietnam native *I. tenuipes* (Peck.) Samson.

Introduction

Entomogenous fungi (EPF) are a group of fungi that are very rich in genus and species. They include 12 classes in six phyla of the fungal kingdom. Fungi that cause arthropods are found in the subphyla Ascomycota, Zygomycota, Deuteromycota, Oomycota, and Chytridiomycota. Insect parasitic fungi are classified into two main groups: Arbitrary parasites: are parasitic species that attack and grow on or in a living organism but are also capable of surviving and reproducing on non-living things. This group of fungi can easily be mass-produced on an artificial medium, so they are very popular for commercialization. The obligate parasites, such as Entomophthora spp., require a suitable living host for survival and are often highly specific, making them ideal for IPM in that they are absent. The potential to negatively affect non-target organisms. However, the mass production of these fungi is complicated because they require a living host [1]. Some entomopathogenic fungi have been interested because they can produce multiple secondary metabolites with bioactivities that have the potential for medicines or nutrients. On the other hand, they have been used for bio-control. Nowadays, EPF are also being tested for use in other fields of biotechnology, e.g., nanoparticle biosynthesis and bioconversion of steroids or flavonoids, which are important from an economic point of view [2]. Therefore, the researching the diversity and biological compounds from them are increasing. *I. tenuipes* is the most common species of the genus *Isaria*. They are also called *Paecilomyces tenuipes* [3] or *Isaria japonica* [4], and it is an anamorph of *Cordyceps takaomontana* [2]. The fruiting body and mycelium contain many secondary compounds including contains sugar, amino acids, fatty acids, adenosine, cordycepin, tenuipesine, sterols, trichothecanes, cyclopeptides, pseudo-dipeptide hanasanagin, and isariotins [5]. Many studies have shown them to have pharmacological activities such as anticancer, anti-inflammatory, immune-enhancing, hypoglycemic, and protective neurons against free radical-induced cytotoxicity, steroidogenic, and antioxidant activities [6,8]. They have

been researched and cultivated in Japan, Korea, and China. [9,14].

In Vietnam, *Isaria tenuipes* (Peck.) Samson was first discovered and described by researchers from the Vietnam Academy of Forestry Sciences with 45 samples distributed in Hoang Lien Son National Park. In 2010 - 2011, the research team of Microbiology Technology Department of Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology collected samples belonging the genus *Isaria* in Lamvien plateau. DL0099 is one of 11 specimens of the species *I. tenuipes* (Peck.) Samson was found at Langbiang Mountain, Lamdong Province [15]. The mycelia growth and fruiting body formation are influenced by various physical and chemical factors including temperature, pH value, nutrients, aeration, and others. Carbon and nitrogen sources are essential nutrients for fungal growth because fungi are heterotrophic. The optimization of carbon and nitrogen sources will promote the growth of fungi, contributing to optimal efficiency in mushroom production. Therefore, this study was carried out to evaluate the effects of carbon and nitrogen sources on fungal growth. Then select suitable carbon and nitrogen sources for fungal growth. The results will be beneficial in biomass production and fruit body formation of the Vietnam native *I. tenuipes* (Peck.) Samson.

Materials and methods

Materials

Fungal strain: Isaria tenuipes Peck. (DL0099) was collected from Langbian Mountain, Lamvien Plateau, Vietnam and identified by Dr. Truong Binh Nguyen from Tay Nguyen Institute for Scientific Research, VAST [15] and were deposited at the Department of Microbiology, Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology (VAST). They were maintained in potato dextrose agar medium (PDA) at 4°C.

Chemicals: The potatoes were bought from a merchant in Dalat, Lam Dong. (Potatoes are used to do potato extract for PDA medium). The different chemicals (peptone, yeast extract, malt extract saccharose, glucose, lactose, maltose, $NaNO_3$, $(NH_4)_2SO_4$) and instruments used were obtained from different companies in Taiwan and some were imported from Japan, the United States, and Germany.

Experimental design

The experiments were conducted at the Department of Microbiology, Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology (VAST) in Vietnam. The experiments were arranged in a randomized complete design with three replications per treatment.

Strains activation

PDA medium (potato extract: 200 g/L; glucose: 20 g/L; agar: 15 g/L) was autoclaved for 15 minutes at 121^{o}C and then distributed into Petri dishes under sterile conditions. DL0099 mycelium was taken out of the refrigerator and placed at room temperature for 24 hours, then the mycelium (0.8 cm diameter) was transferred to the medium dishes prepared above, incubated in dark for 10 days. The healthy, disease-free seed plates were used for next experiments.

The effects of different carbon sources on DL0099 growth

Potato agar (PA) medium containing different carbohydrate sources (glucose, saccharose, lactose, maltose, and sucrose) at a 20 g/L concentration was used for the experiment of carbohydrate sources. The PA medium containing 200 g of potato, 15 g of agar powder, and 1000 mL of distilled water was used as the control treatment. The media and petri dishes were autoclaved at 121°C (at a pressure of 1.3 kg/cm2) for 15 Min. The mycelium discs (1-cm diameter) of *Isaria tenuipes* was placed in medium plates (15-mL medium) under aseptic condition and incubated at 25°C in the darkness. The diameter of the mycelium expansion was measured 7 days after inoculation. Mycelium density and fruit body formation were noted.

The effects of different glucose concentrations on DL0099 growth

Use the appropriate carbon source in the above experiment to arrange this experiment with concentrations of 10; 20; 30; 40; and 50 g/L. The media and Petri dishes were autoclaved at 121°C (at a pressure of 1.2 kg/cm^2) for 15 min. The mycelium discs (1 cm in diameter) of the DL0099 strain were placed in the centers of medium plates (10 mL medium) under aseptic conditions. For each concentration of 5 Petri dishes, repeat the experiment 3 times. Incubated conditions at 25°C in darkness.

The diameter of the mycelium expansion was measured 7 days after inoculation. Mycelium density and fruit body formation were noted.

The effects of different nitrogen sources on DL0099 growth

Potato glucose agar (PDA) medium containing different nitrogen sources such as yeast extract, peptone, malt extract, $NaNO_3$, and $(NH_4)_2SO_4$ at a 5 g/L concentration was used for the experimentation of nitrogen sources. A PDA containing 200 g of potato, 15 g of agar powder, and 1000 mL of distilled water was used as the control treatment. The mycelium discs (1 cm in diameter) of the DL0099 strain were placed in the centers of medium plates (10 mL medium) under aseptic conditions and incubated at 25^{o}C in the darkness. The diameter of the mycelium expansion was measured 7 days after inoculation. Mycelium density and fruit body formation were noted.

The effects of different nitrogen concentrations on DL0099 growth

In this experiment, peptone was used to investigate the optimal concentration for the mycelium expansion and

mycelium density. Malt extract was used to investigate the optimal concentration for the formation of fruiting bodies of DL0099 at the following concentrations: 3; 6; 10; 15, and 20 g/L. After the media and Petri dishes were sterilized, the mycelium discs of 1 cm in diameter of the DL0099 strain were placed in the centers of medium plates (10 mL medium) under aseptic conditions and incubated at 25°C in darkness. The diameter of the mycelium expansion was measured 7 days after inoculation. Mycelium density and primordial initiation were noted.

Statistical analysis

Data were analysed in the Statistical Package for Social Sciences (SPSS) version 16 using one-way analysis of variance (ANOVA) and Tukey test to analyse the significant treatment comparison at 5% level of significance. The mean and standard deviation are used to express each value. All the experiments were conducted in triplicates.

Results

Effects of different carbon sources

To determine the suitable carbon source for the mycelium growth, DL0099 mycelium was grown in PA medium supplemented with different sugars: sucrose, glucose, lactose, saccharose maltose, and PA was control medium The experimental results are shown in Table 1 and Figure 1.

The results showed that *I. tenuipes* (Peck.) Samson (DL0099) can grow on carbon sources such as sucrose, glucose, lactose, galactose, and maltose. Compared with the control medium, carbon sources had a positive effect on the mycelium expansion and density. Among the used carbon sources, glucose was the best for the mycelium growth and density. The colony diameter was 4.18 cm after 7 days of inoculation. The mycelium expansion and density were the worst on medium containing lactose. The mycelium growth was no significant difference among sucrose, sucrose, and maltose. Fruit body formation was not observed after 20 days of inoculation in the sugars used. Thus, under these experimental conditions, glucose was considered a suitable carbon source for the growth of the mycelium *I. tenuipes* (Peck.) Samson (DL0099). It was chosen as a carbohydrate source in the following test.

Effect of different glucose concentrations

From the above results, glucose was used to investigate the carbon concentration suitable for the DL0099 growth of the mycelium at the following concentrations: 0; 10; 20; 30; 40; 50 g/L. The results are shown in Table 2 and Figure 2.

According to statistical results, there was no significant difference in colony diameter after 7 days of inoculation when using glucose concentration of 20~40 g/L. Glucose concentrations from 20 to 40 gave the highest mycelium colony diameter (4.12~4.18 cm) and higher than the PA medium (control) and other glucose concentrations. The experimental results also showed that there was no fruit body formation at the glucose concentration of 0~10 g/L, but at the concentration from 20~50 g/L, the yellow pigment formed was quite pale. Thus, in this experimental condition, the appropriate glucose concentration for the DL0099 growth was 20~40 g/L.

Table 1. Effect of different carbon on the growth of DL0099.

Carbohydrate sources	Mycelium colony diameter for 7 days after inoculation (cm)	Mycelium density	Fruit body formation
Saccharose	4.13 ± 0.29^{ab}	SC	-
Sucrose	4.12 ± 0.10^{ab}	SC	-
Lactose	3.90 ± 0.19^{cd}	T	-
Glucose	4.18 ± 0.16^{a}	C	-
Maltose	4.00 ± 0.00^{abc}	SC	-
Control	3.83 ± 0.06^{d}	ST	-

Means (X) within the same column followed by the same letters are not significantly different at p< 0.05. ST: somewhat thin; SC: Somewhat compact; C: compact; T: Thin; (-): No fruit body formation.

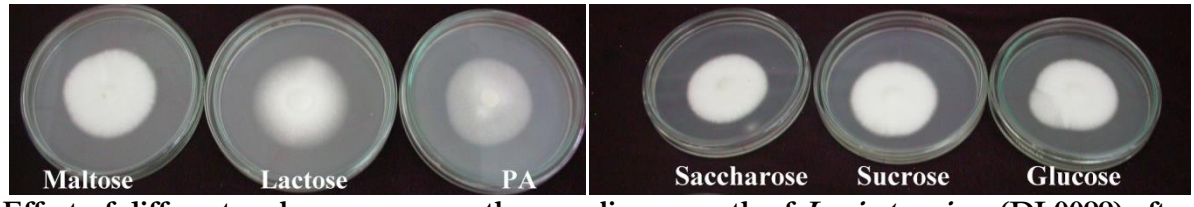

Figure 1. Effect of different carbon sources on the mycelium growth of *Isaria tenuipes* (DL0099) after 7 days of inoculation.

Table 2. Effects of different glucose concentration on the growth of DL0099.

Glucose concentration (g/L)	Mycelium colony diameter for 7 days after inoculation (cm)	Mycelium density	Fruit body formation for 20 days after inoculation
0	3.8 ± 0.1^{d}	SC	-
10	4.05 ± 0.09^{abc}	SC	-
20	4.17 ± 0.15^{ab}	ST	-
30	4.18 ± 0.16^{ab}	SC	-
40	4.12 ± 0.21^{ab}	SC	-
50	3.85 ± 0.05^{cd}	T	-

Means (X) within the same column followed by the same letters are not significantly different at p< 0.05. ST: somewhat thin; T: Thin; SC: Somewhat compact; (-): No fruit body formation.

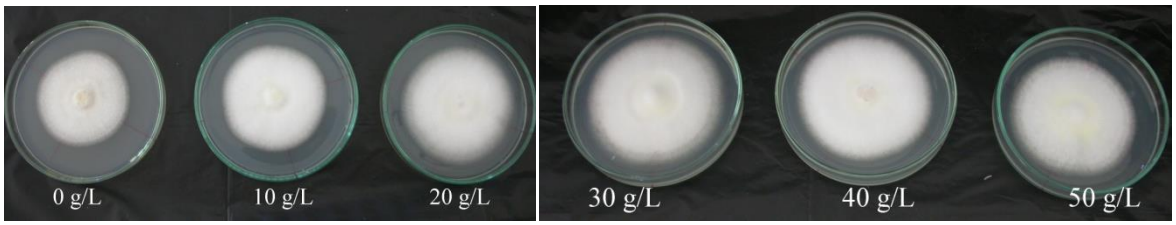

Figure 2. Effect of different glucose concentration on the mycelium growth of *Isaria tenuipes* (DL0099).

Effects of different nitrogen sources

This experiment was conducted based on PDA medium supplemented with 10 g/L of organic nitrogen sources including peptone, malt extract, and yeast extract; inorganic nitrogen sources including $(NH_4)_2SO_4$, and $NaNO_3$. The data on the effects of different nitrogen sources on the DL0099 growth are shown in Table 3, Figure 3.

DL0099 mycelium can grow on both inorganic and organic nitrogen sources, but mycelium grows better on organic nitrogen sources. There were significant differences in mycelium density between organic and inorganic nitrogen sources. Mycelium density occurred in the medium containing organic nitrogen was more compact than that in the medium using inorganic nitrogen. Mycelium density was best on medium supplemented with peptone. There was not a significant difference in mycelium colony diameter among inorganic nitrogen sources and organic nitrogen sources. Seven days after inoculation, the mycelium colony diameters of DL0099 were 4.03~4.2 cm in media containing nitrogen sources. After 20 days of inoculation, DL0099 had fruiting body formation on the medium supplemented with organic nitrogen sources (malt extract, yeast extract, peptone). However, this was not found on media containing inorganic nitrogen sources ($NaNO_3$, $(NH4)_2SO_4$). In particular, among organic nitrogen sources, the fruiting body formation was best on medium supplemented with malt extract. Therefore, depending on the purpose of the culture, we choose suitable nitrogen sources. This experiment showed that peptone was a suitable nitrogen source for mycelium density and malt extract was a suitable nitrogen source for fruit body formation.

Effect of different nitrogen concentration

From the results of the above experiment, peptone was used to investigate the optimal concentration for the mycelium expansion and mycelium density. Malt extract was used to investigate the optimal concentration for the formation of fruiting bodies of DL0099 at the following concentrations: 3; 6; 10; 15, and 20 g/L. The results are shown in Table 4 and Figure 4.

The results in Table 4 showed that the peptone concentration had a significant effect on the mycelium density and colony diameter. The mycelium density and colony diameter were increase when the peptone concentration from 3 to 10 g/L. On PDA medium without peptone addition, colony diameter was 3.82~4.30 cm after 7 days of incubation, no fruit body formation after 20 days of inoculation. However, on medium supplemented with 10 g/L peptone, the colony diameter was 4.3 cm after 7 days of culture. The mycelium colony diameter decreased when the peptone concentrations was 15-20 g/L. When investigating the effect of malt extract concentration on fruiting body formation, we found that fruit body formation was promoted when the malt extract concentration was increased from 3 to 10 g/L. The fruit body formation was best in medium supplemented with 10 g/L malt extract. In this experimental condition, the suitable peptone concentration for the mycelium density and colony diameter was 10 g/L.

Table 3. Effect of different nitrogen sources on the growth of DL0099.

Nitrogen sources	Mycelium colony diameter for 7 days after inoculation (cm)	Mycelium density	Fruit body formation for 20 days after inoculation
$NaNO_3$	4.03± 0.029[a]	T	-
$(NH_4)_2SO_4$	4.10 ± 0.20[a]	T	-
Peptone	4.20 ± 0.03[a]	C	+
Yeast extract	4.15 ± 0.05[a]	C	+
Malt extract	4.12 ± 0.06[a]	C	++

Means (X) within the same column followed by the same letters are not significantly different at p< 0.05. T: Thin; C: compact; (-): No fruit body formation, (+): fruit body formation.

Figure 3. Effect of different nitrogen sources on the mycelium growth of *Isaria tenuipes* (DL0099).

Table 4. Effect of different peptone concentration on the mycelium growth of DL0099.V

Peptone concentration (g/L)	Mycelium colony diameter for 7 days after inoculation (cm)	Mycelium density
3	4.18 ± 0.03[ab]	SC
6	4.23 ± 0.06[ab]	C
10	4.30 ± 0.26[a]	C
15	4.07 ± 0.12[c]	C
20	3.82 ± 0.21[d]	C

Means (X) within the same column followed by the same letters are not significantly different at p< 0.05. C: compact; SC: Somewhat compact.

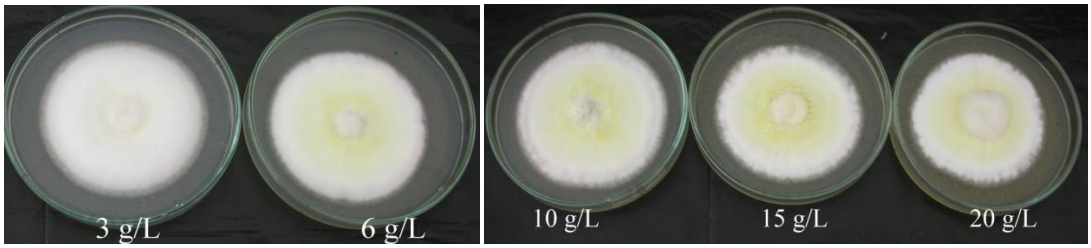

Figure 4. Effect of different peptone concentration on the mycelium growth of *Isaria tenuipes* (DL0099).

Discussion

The mycelia growth and fruiting body formation are influenced by various physical and chemical factors including temperature, pH value, nutrients, aeration, and others. Carbon sources are very important for every organism. Mushrooms use carbohydrates to build cell structure, metabolism, and energy in cells and the body. For different types of mushrooms, the carbohydrate requirements for their survival are also different. Because of their morphological and structural characteristics, they use a variety of carbohydrate sources. Having one or several sources of carbohydrates will suit the growth of certain fungi. Fu et al., (2017) reported that sucrose was the best carbohydrate source for the mycelium growth of fungus *Villosiclava virens* [16]. Neelam et al., (2011) also indicated dextrose was the best carbohydrate source for mycelium growth of the *Pleurotus florid* [17]. Mao et al., (2019) demonstrated that glucose was an optimal carbohydrate source for cordycep in production of the Chinese traditional medicinal mushroom *Cordyceps militaris*. Moreover, glucose was also identified as the best carbohydrate source for exopolysaccharide production of edible mushrooms such as *Phellinus* [18]. The results in the table 1 and 2 showed that *Isaria tenuipes* (Peck.) Samson (DL0099) can grow on the carbon sources: Saccharose, glucose, lactose, and maltose. In which, 20~40 g/L glucose was the best for the DL0099 growth. The colony diameter was 7.02 cm after 7 days of inoculation. The study of Xu et al., (2006) also showed that the suitable carbon source for the growth of the

mycelium Paecilomyces tenuipes C240 was glucose [19]. This result is similar to the present study.

Fungi use nitrogen to form the mycelium membrane in the form of chitin compounds. In addition, nitrogen also participates in the structural components of the cell in the form of proteins, enzymes, coenzymes, etc. integral part of the fungus. Fungi can use different nitrogen sources but not all nitrogen sources are equally suitable for all fungi. The selection of an appropriate nitrogen source is very important to achieve high growth quality of mycelium and the fruit bodies formation. In this study, DL0099 mycelium can grow on both inorganic and organic nitrogen sources, but mycelium grows better on organic nitrogen sources. There were significant differences in mycelium density between organic and inorganic nitrogen sources. Mycelium density occurred in the medium containing organic nitrogen was more compact than that in the medium using inorganic nitrogen. These results are similar to those previously reported in other insect parasitic fungi species [20,21]. Mycelium density was best on medium supplemented with peptone 10 g/L. In addition, we found that among the organic carbon sources used, 10 g/L malt extract best promoted the best fruit body formation. The research results are the basis for propagation and cultivating DL0099 strain with high efficiency.

Conclusion

This study investigated the effects of carbon and nitrogen sources on the mycelium growth and fruiting body formation of *I. tenuipes* (Peck.) Samson (DL0099) from Lamdong, Vietnam. According to the results, the different carbon (glucose, sacharose, lactose, maltose, sucrose) and nitrogen sources (yeast extract, peptone, $NaNO_3$, KNO_3, $(NH_4)_2SO_4$) positively affected to the mycelium density, mycelium colony diameter, and fruit body formation. Glucose with a concentration of 20~40 g/L was suitable for the growth of DL0099. Organic nitrogen sources (peptone, malt extract, and yeast extract) were suitable for the growth of *I. tenuipes* (DL0099), in which peptone 10 g/L was the most suitable for the mycelium density and colony diameter. The fruit body formation was best in the medium supplemented with 10g/L malt extract. The mushroom growers can use the results as a guideline to effectively cultivate *I. tenuipes* (DL0099).

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Conflict of Interests

None

Author Contribution

Conceived idea and designed the experimental study, performed the experiments, wrote, and format the manuscript, approved the final version for submission.

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